

Kinetics of infection of plasma, blood leucocytes and lymphoid tissue from Atlantic salmon *Salmo salar* experimentally infected with pancreas disease

Gillian Houghton

SOAFD Marine Laboratory, PO Box 101, Victoria Road, Aberdeen AB9 8DB, Scotland, UK

ABSTRACT: Plasma, blood leucocytes, splenocytes and kidney homogenates were all shown to be infective following intraperitoneal injection of Atlantic salmon post-smolts with pancreas disease infective kidney homogenate. The kinetics of infectivity were temperature dependent with a more rapid dissemination of infection at 14°C compared to 9°C and 6°C. At all 3 temperatures, the plasma remained highly infectious from Day 1 post injection until pancreas pathology started to occur at which time it became non-infectious. The blood leucocytes, splenocytes and kidney became infective after the plasma, the time interval being temperature dependent but, as with the plasma, these cells and tissues became non-infectious when peak pathology occurred.

KEY WORDS: Pancreas disease · Kinetics of infection · Atlantic salmon · Plasma · Leucocytes · Kidney

INTRODUCTION

Pancreas disease (PD) occurs in farmed Atlantic salmon during the sea water phase of their culture (Munro et al. 1984, Murphy et al. 1992). The main pathology is the total necrosis of the acinar cells of the exocrine pancreas.

While the aetiological agent of the disease is unknown, the disease is transmissible by injection of kidney or spleen and the agent will pass through a 0.22 µm filter and thus is believed to be a virus (McVicar 1987, 1990, Raynard & Houghton 1993). That the disease can be transmitted using kidney and spleen homogenates suggests that the causative agent may be present in one or more types of fish leucocytes as has been found with infectious pancreatic necrosis virus (IPNV) in rainbow trout *Oncorhynchus mykiss* leucocytes (Swanson & Gillespie 1982, Yu et al. 1982, Saint-Jean et al. 1991) and Atlantic salmon leucocytes (Knott & Munro 1986) and more recently with infectious salmon anaemia (ISA) in Atlantic salmon leucocytes (Dannevig & Falk 1994, Dannevig et al. 1994, Hovland et al. 1994). In order to elucidate where the

PD agent may replicate, it is first necessary to investigate the kinetics of infection and the cells involved.

The present study was designed to examine blood products and lymphoid tissue for the causative agent of pancreas disease following exposure of Atlantic salmon to an experimental infection.

MATERIALS AND METHODS

Fish. Atlantic salmon were maintained at the fish cultivation unit of the Marine Laboratory, Aultbea, Wester Ross, or at the Marine Laboratory, Aberdeen, Scotland. All fish were reared at the fish cultivation unit which has a history of being free of PD and IPNV. In addition, fish were tested prior to experimentation to ensure the absence of these 2 diseases.

Fish used to follow the kinetics of infection (donors) were post-smolts maintained either in fresh water (FW) or sea water (SW) in 1 m diameter tanks at a flow rate of 6 l min⁻¹ (FW) or 10 l min⁻¹ (SW). Infection kinetic experiments were conducted at 3 different water temperatures, namely 14, 9 or 6°C. Fish used to test infec-

tivity of the various samples (recipients) were parr maintained in fresh water in 1 m diameter tanks at a flow rate of 5 l min⁻¹. Fish were fed a standard commercial diet by automatic feeders to satiation (Mainstream diets, BP nutrition). Fish were acclimated in tanks 2 to 4 wk prior to experimentation. Prior to injection, fresh water fish were anaesthetised in ethyl-4-aminobenzoate (benzocaine, Sigma, UK) dissolved in ethanol and sea water fish were anaesthetised in 3-aminobenzoic acid ethyl ester (MS222, Sigma, UK).

Histology. For histological examination of the pancreas, pyloric caeca with pancreas were fixed in 20% buffered formol saline, embedded in paraffin wax and 5 µm sections cut and stained with haematoxylin and eosin. Assessment of PD pathology was carried out according to Raynard & Houghton (1993).

Preparation of PD infective and control kidney homogenates. PD infective and control normal kidney homogenates for injecting the donor fish were prepared as described by Raynard & Houghton (1993). Fish were injected with a dose of 6 µg protein homogenate g⁻¹ body weight. The protein concentration was determined following procedure 690-A (Sigma).

Primary infection. The Atlantic salmon used for each of the experiments were of a range of weights and numbers. Fish kept at 14°C (FW) had a mean weight of 128.4 ± 14.8 g (n = 56), fish kept at 9°C (SW) had a mean weight of 225.6 ± 52.4 g (n = 48) and fish kept at 6°C (FW) had a mean weight of 87.6 ± 21.4 g (n = 80). The fish used in the 14 and 6°C experiments were from the same stock. Fish were anaesthetised and half of each group of fish given an intraperitoneal injection of 0.2 ml of PD infective kidney homogenate and the other half of each group given an i.p. injection of negative control homogenate. PD and control fish were kept in separate tanks. Fish were sampled at the following days post-infection (p.i.): 1, 3, 5, 7, 11, 15 and 24 for the fish kept at 14°C; 1, 3, 5, 12, 19 and 27 for the fish kept at 9°C and 1, 3, 7, 11, 15, 20, 24, 27, 33 and 38 for the fish kept at 6°C. At each sample time, 6 to 8 PD infected fish and 6 to 8 non-infective controls were sampled.

Sampling of blood and leucocyte separation. Following anaesthetisation, blood samples were collected from the caudal vein into heparinised vacutainer tubes. For plasma, blood was centrifuged at 400 × g for 5 min. The plasma fraction was collected, pooled, frozen on dry ice and stored at -80°C. For blood leucocytes, the buffy coat and red blood cells were collected and resuspended at a ratio of 1:4 (v/v) in Leibovitz (L-15, Gibco) medium supplemented with heparin (10 units ml⁻¹), antibiotics (100 IU ml⁻¹ penicillin; 100 µg ml⁻¹ streptomycin) and 2% Myoclon foetal calf serum (heat inactivated at 56°C for 30 min; FCS, Gibco) (L-15 media 1). The diluted blood was layered onto a 51%

Percoll density gradient (Pharmacia). After centrifugation at 400 × g for 30 min at 4°C, a leucocyte fraction was obtained at the interface between the Percoll and the medium. Samples still contaminated with red blood cells were re-processed. The leucocytes were washed in L-15 supplemented with 0.1% FCS, antibiotics and without heparin (L-15 media 2) for 8 min at 400 × g and resuspended in L-15 supplemented with 5% FCS and antibiotics (L-15 media 3). Cell counts were made in a haemocytometer and samples pooled at a concentration of 1.5 × 10⁷ cells ml⁻¹, frozen on dry ice and stored at -80°C.

Isolation of splenocytes. Spleens were removed from fish and placed in L-15 media 1. Splenocytes were isolated by pressing the spleens through a stainless steel metal grid. The cells were then layered onto a 51% Percoll gradient and the same procedure carried out as for the blood leucocytes. Samples were pooled to give a concentration of 1.5 × 10⁷ cells ml⁻¹, frozen on dry ice and stored at -80°C.

Preparation of kidney homogenates. Whole kidneys were collected from each fish, pooled, frozen on dry ice and stored at -80°C. Frozen kidneys were later thawed, homogenised in phosphate-buffered saline (PBS, w/o Ca⁺⁺, Mg⁺⁺) pH 7.2 following the method of Raynard & Houghton (1993) and re-frozen. The dose used was determined by measuring the protein concentration following procedure 690-A (Sigma).

Testing for infectivity of plasma, blood leucocytes, splenocytes and kidney homogenates. Atlantic salmon parr were used for the testing of samples and were injected i.p. at a volume of 0.1 ml (n = 30 to 50). Plasma was diluted 1:3 in PBS pH 7.2 (w/o Ca⁺⁺, Mg⁺⁺). Splenocytes and blood leucocytes were diluted 1:2 with PBS and kidney homogenates injected at a dose of 20 µg g⁻¹ body weight. Samples of pyloric caeca and pancreas were taken from 10 fish in each group at each sample time. The recipient fish were kept at ambient temperature which varied throughout the year. As lower temperature prolongs the development of the pathology (Raynard & Houghton 1993), sampling was extended (up to 9 wk) in order to cover the peak prevalence of pancreas disease pathology at low temperatures.

RESULTS

Terminology

'Early PD': vacuolation and rounding of the acinar cells over the whole exocrine tissue. Absence of zymogen from many cells.

'Acute PD': total loss of the acinar cells of the exocrine pancreas.

Kinetics of infectivity at 14°C

The percentage of donor fish showing PD pathology is shown in Fig. 1a. From Days 1 to 5 p.i., the exocrine pancreas showed a normal histological appearance with no acinar cell loss. On Days 7 and 11, early signs of PD pathology could be seen with rounding of the acinar cells. This developed into acute PD pathology with 75% of the donor fish showing total acinar loss on Days 15 and 24. At no time did the control fish show any signs of PD pathology (results not shown).

PD infectivity was detectable at high levels in plasma from Days 1 to 11 with up to 100% of the recipient fish developing acute PD from Days 5 to 11 post infection after which infectivity decreased with no infectivity being detected on Day 24 (Fig. 1b).

Blood leucocytes (Fig. 1c) showed no infectivity on Day 1 with a very low level of infectivity being detectable on Day 3. Higher levels of infectivity occurred from Day 5 to 11 after which infectivity fell to a low level on Day 15 becoming negative on Day 24.

Splenocytes (Fig. 1d) and kidney homogenates (Fig. 1e) showed a similar pattern with low levels of infectivity on Day 3 where 30 to 40% of recipient fish developed acute PD increasing to between 80 and 100% of recipient fish developing acute PD when injected with splenocytes taken on Days 5, 7 and 11 and kidney homogenates taken on Days 5, 7, 11 and 15. The level of infectivity of splenocytes was reduced

on Day 15 becoming negative on Day 24. A very low level of infectivity occurred in kidney homogenates taken at Day 24 with only 10% of recipient fish developing acute PD.

A summary of the results is given in Table 1a. At no time did plasma, blood leucocytes, splenocytes or kidney homogenates taken from non-infected control fish show any infectivity (results not shown).

Kinetics of infectivity at 9°C

The percentage of donor fish showing PD pathology can be seen in Fig. 2a. From Days 1 to 12, the exocrine pancreas showed no acinar cell loss. On Day 19, acute PD pathology could be seen in 16% of the donor fish, increasing to 66% of donor fish showing total loss of acinar cells on Day 27. At no time did the control fish show any signs of PD pathology (results not shown).

PD infectivity was detectable in plasma (Fig. 2b) at low levels on Days 1 and 3 reaching high levels from Days 5 to 19 where up to 100% of the recipient fish developed acute PD. On Day 27 no infectivity was detected in the plasma.

Blood leucocytes (Fig. 2c) showed no infectivity on Days 1 and 3 with a low level of infectivity being detectable on Day 5. Higher levels of infectivity occurred on Days 12 and 19 becoming negative on Day 27.

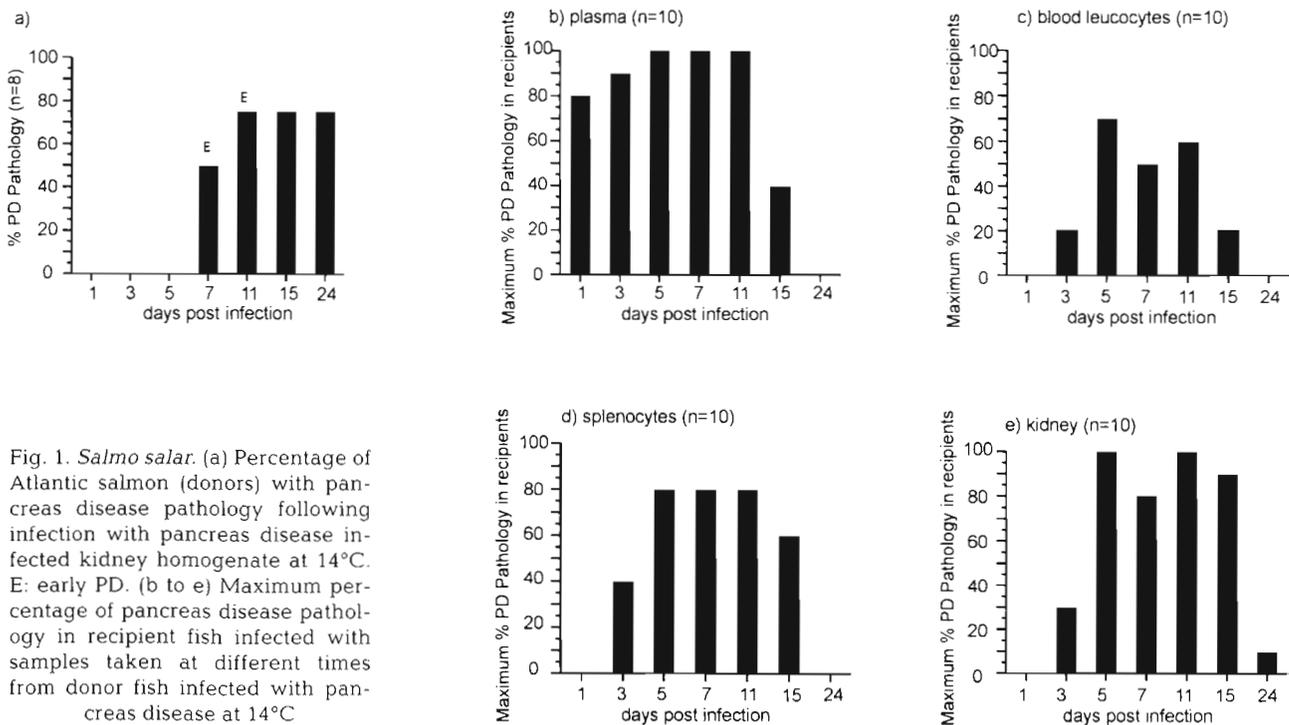


Fig. 1. *Salmo salar*. (a) Percentage of Atlantic salmon (donors) with pancreas disease pathology following infection with pancreas disease infected kidney homogenate at 14°C. E: early PD. (b) to (e) Maximum percentage of pancreas disease pathology in recipient fish infected with samples taken at different times from donor fish infected with pancreas disease at 14°C

Table 1 *Salmo salar*. Summary of infectivity of plasma, blood leucocytes, splenocytes and kidney homogenates taken at different times from donors experimentally infected at: (a) 14°C, (b) 9°C, (c) 6°C. Percentage PD of recipients: -, 0%; +, ≤40%; ++, 41 to 70%; +++, ≥71%. E: early PD

(a) 14°C		Days post-injection								
Group	1	3	5	7	11	15	24			
Plasma	+++	+++	+++	+++	+++	+	-			
Blood leucocytes	-	+	+++	++	++	+	-			
Splenocytes	-	+	+++	+++	+++	++	-			
Kidney	-	+	+++	+++	+++	+++	+			
Controls	-	-	-	-	-	-	-			
% PD of donors ^a	0	0	0	50E	75E	75	75			
(b) 9°C		Days post-injection								
Group	1	3	5	12	19	27				
Plasma	+	+	+++	+++	+++	-				
Blood leucocytes	-	-	+	+++	++	-				
Splenocytes	-	-	-	+++	+	-				
Kidney	-	-	-	+++	+	-				
Controls	-	-	-	-	-	-				
% PD of donors ^a	0	0	0	0	16	66				
(c) 6°C		Days post-injection								
Group	1	3	7	11	15	20	24	27	33	38
Plasma	+++	+++	+++	+++	+++	+++	+++	+++	-	-
Blood leucocytes	-	-	-	+	+	+++	++	+	-	-
Splenocytes	-	-	-	-	+	++	+	++	+	-
Kidney	-	-	+++	++	++	++	++	++	++	-
Controls	-	-	-	-	-	-	-	-	-	-
% PD of donors ^a	0	0	0	0	0	25E	38E	25	63	63

^aPercentage of fish showing early or acute PD

Splenocytes (Fig. 2d) and kidney homogenates (Fig. 2e) showed a similar pattern with no infectivity on Days 1, 3 and 5. Infectivity increased to high levels on Day 12, where 70 to 100% of recipient fish developed acute PD when injected with splenocytes and kidney homogenates respectively. The level of infectivity of both splenocytes and kidney homogenates was reduced on Day 19 becoming negative on Day 27.

A summary of the results is given in Table 1b. At no time did plasma, blood leucocytes, splenocytes or kidney homogenates taken from non-infected control fish show any infectivity (results not shown).

Kinetics of infectivity at 6°C

The percentage of donor fish showing PD pathology can be seen in Fig. 3a. From Days 1 to 15, the exocrine pancreas showed no acinar cell loss. On Days 20 and 24, early signs of PD pathology were evident in 25 and 38% of fish respectively. This developed into acute PD pathology with 63% of the donor fish showing total

acinar loss on Days 33 and 38. At no time did the control fish show any signs of PD pathology (results not shown).

PD infectivity was detectable at high levels in plasma from Days 1 to 27 with up to 100% of recipients developing acute PD pathology after which no infectivity could be detected on Days 33 and 38 (Fig. 3b).

Blood leucocytes (Fig. 3c) showed no infectivity on Days 1, 3 and 7 with low levels of infectivity being detectable on Days 11 and 15. A high level of infectivity occurred on Day 20 where up to 100% of recipient fish developed acute PD after which infectivity decreased on Days 24 and 27 with no infectivity being detectable on Days 33 and 38.

Splenocytes (Fig. 3d) showed no infectivity on Days 1, 3, 7 and 11 with a low level of infectivity occurring on Day 15. Infectivity increased on Days 20, 24 and 27 decreasing on Day 33 and becoming negative on Day 38. Kidney homogenates (Fig. 3e) showed no infectivity on Days 1 and 3 with a high level of infectivity occurring on Days 7, 11, 15, 20 and 24 where 70 to 90% of recipient fish developed acute PD. Infectivity began to decrease on Day 33 becoming negative on Day 38.

A summary of the results is given in Table 1c. At no time did plasma, blood leucocytes, splenocytes or kidney homogenates taken from non-infected control fish show any infectivity (results not shown).

DISCUSSION

The results presented here lend further support for a viral aetiology for the causative agent of pancreas disease in Atlantic salmon as proposed by McVicar (1990) and Raynard & Houghton (1993). The spread of the infectious agent through the circulatory system to the lymphoid tissues is typical of a viraemia as has been shown with other viruses in fish such as infectious pancreatic necrosis virus (IPNV) (Swanson & Gillespie 1982), infectious haematopoietic necrosis virus (IHNV) (Yamamoto & Clermont 1990, Drolet et al. 1994) and infectious salmon anaemia (ISA) (Dannevig et al. 1994).

That the time taken for peak pathology to occur in the donor fish and dissemination of the causative agent of PD through the fish is affected by temperature also

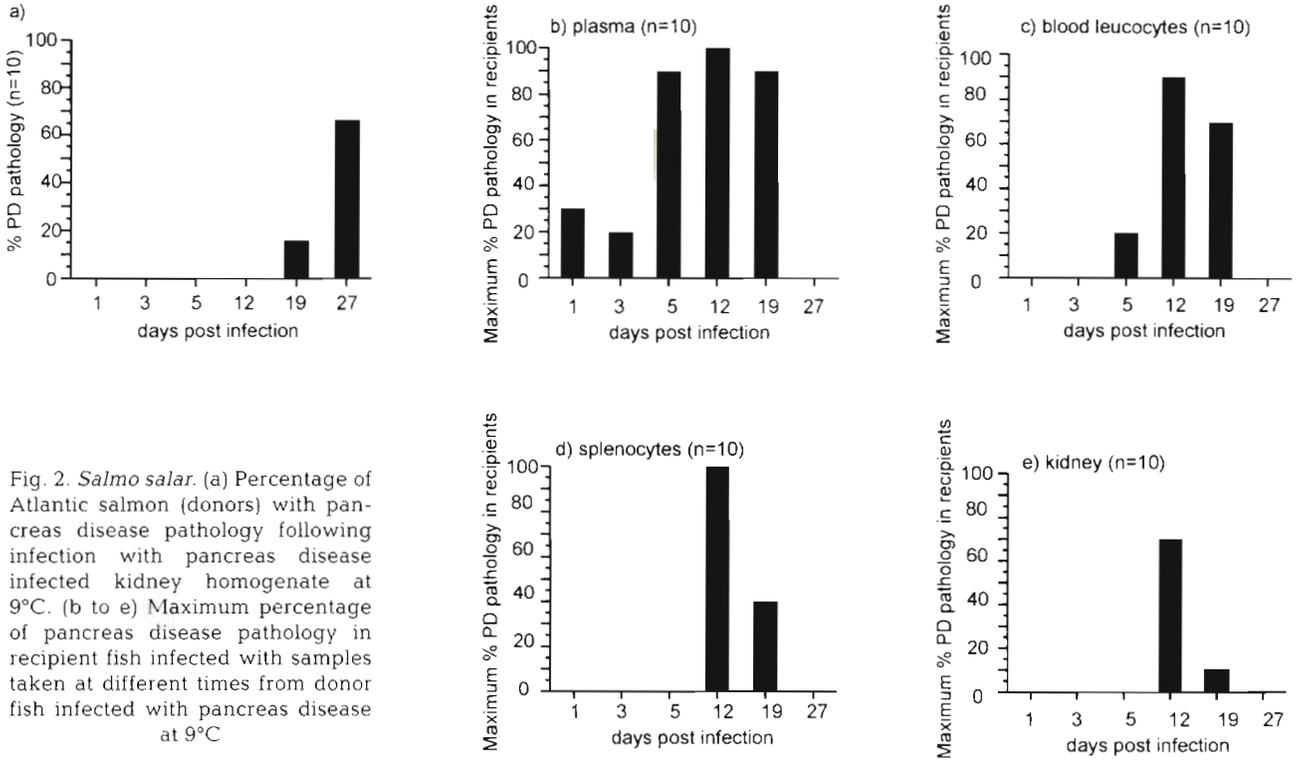


Fig. 2. *Salmo salar*. (a) Percentage of Atlantic salmon (donors) with pancreas disease pathology following infection with pancreas disease infected kidney homogenate at 9°C. (b to e) Maximum percentage of pancreas disease pathology in recipient fish infected with samples taken at different times from donor fish infected with pancreas disease at 9°C

suggests a viral infection. This confirms earlier results by Raynard & Houghton (1993) and Houghton (1994) where the time taken for peak pathology to occur following experimental infection of fish was affected by temperature with the lower the temperature the later the time at which acute PD pathology occurred.

Although the fish infected at 9°C were post-smolts in sea water and therefore are not directly comparable with the fish infected at 14 and 6°C in fresh water which occurred concurrently, the results are consistent with the trend of lower temperatures increasing the time for PD pathology to occur.

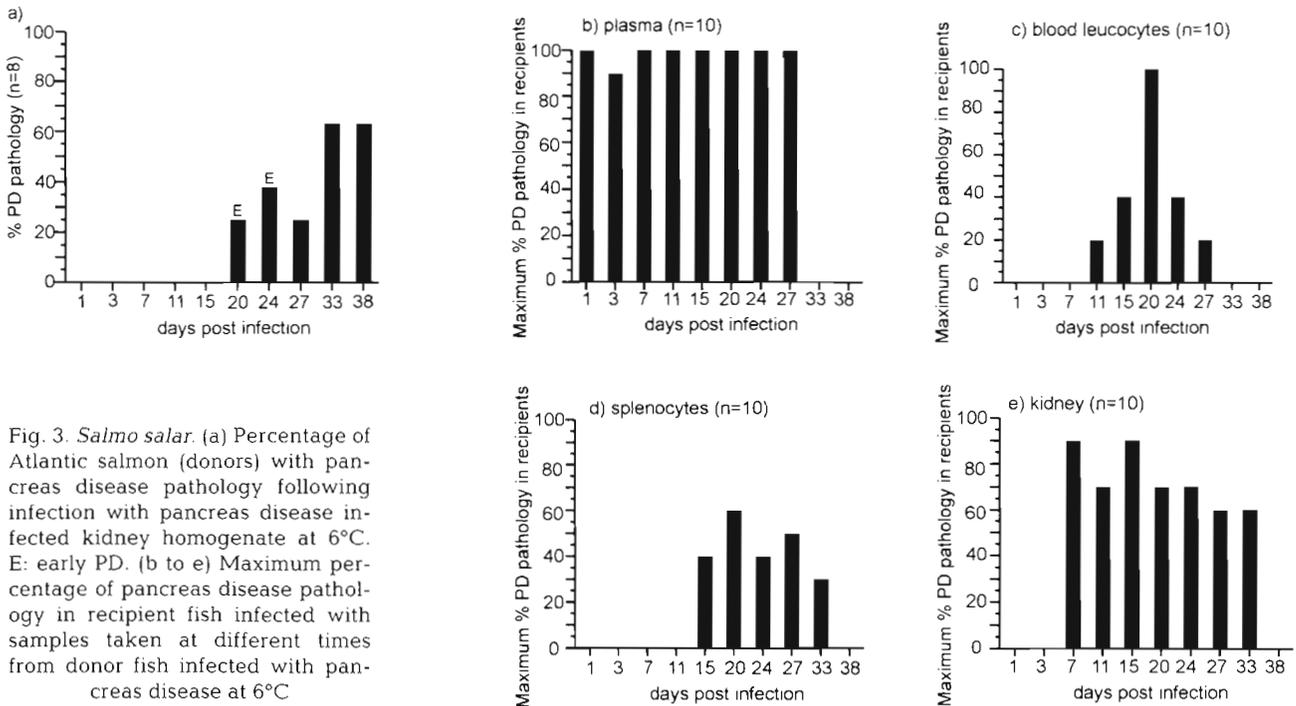


Fig. 3. *Salmo salar*. (a) Percentage of Atlantic salmon (donors) with pancreas disease pathology following infection with pancreas disease infected kidney homogenate at 6°C. E: early PD. (b to e) Maximum percentage of pancreas disease pathology in recipient fish infected with samples taken at different times from donor fish infected with pancreas disease at 6°C

Following intraperitoneal injection of the donor fish with PD infective kidney homogenate, the PD agent occurred in the circulatory system at all 3 temperatures very soon after the fish were injected. High levels of infectivity were found in the plasma from Day 1 onwards and initially, this would be due to the primary inoculation. As time progressed the infectivity was still maintained at high levels throughout the sampling of the donor fish until peak pathology occurred, suggesting that replication and release of the infectious agent into the circulatory system from target cells must be occurring. It would be via the circulatory system that haematopoietic tissue and lymphoid organs would become infective. Following high levels of infectivity in the plasma, the infectious agent could be detected in the peripheral blood leucocytes, splenocytes and kidney starting at low levels but increasing to high levels, suggesting viral replication. The kidney homogenate contained both anterior and posterior kidney. The anterior kidney contains large numbers of leucocytes although the preparations would also be contaminated with blood products unlike the splenocytes which were washed cells. These results suggest that leucocytes may be one of the main target cells of the causative agent of PD. However, it is not known which type of leucocyte the PD agent may be infecting or whether the leucocytes are also a site of replication of the PD agent.

One of the problems in diagnosing pancreas disease is that the only method available is by lethal sampling of fish for histological examination of the pancreas with the disease only being confirmed if there is total acinar cell loss. At this stage, fish may already be severely affected. Thus early diagnosis before acute PD is reached is important since it will allow time for steps to be taken such as withholding of food which may alleviate the full effect of the disease (R. Raynard pers. comm.). The finding that plasma remains infective right up to the time when acute PD occurs, which can be up to 27 d at low temperatures, offers the potential for a method for early diagnosis by non-lethal sampling of blood for immunological detection of the PD agent in the plasma. However it is important to establish whether significant levels of the PD agent are found in the plasma in fish infected by waterborne challenge by cohabitation with infected fish.

Another interesting result from the present work is that at all 3 temperatures, when peak pathology occurs, there is a concurrent loss of infectivity in the plasma, blood leucocytes, splenocytes and kidney. The reason for this is unclear but suggests that antibody may play a role in neutralising free virus followed by acinar cell lysis due to antibody and complement as suggested previously by Houghton (1994).

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LITERATURE CITED

- Dannevig BH, Falk K (1994) Atlantic salmon, *Salmo salar* L., develop infectious salmon anaemia (ISA) after inoculation with *in vitro* infected leucocytes. *J Fish Dis* 17:183–187
- Dannevig BH, Falk K, Skjerve E (1994) Infectivity of internal tissues of Atlantic salmon, *Salmo salar* L., experimentally infected with the aetiological agent of infectious salmon anaemia (ISA). *J Fish Dis* 17:613–622
- Drolet BS, Rohovec JS, Leong JC (1994) The route of entry and progression of infectious haematopoietic virus in *Oncorhynchus mykiss* (Walbaum): a sequential immunohistochemical study. *J Fish Dis* 17:337–347
- Houghton G (1994) Acquired protection in Atlantic salmon *Salmo salar* parr and post-smolts against pancreas disease. *Dis aquat Org* 18:109–118
- Hovland T, Nylund A, Watanabe K, Endresen C (1994) Observations of infectious salmon anaemia virus in Atlantic salmon, *Salmo salar* L. *J Fish Dis* 17:291–296
- Knott RM, Munro ALS (1986) The persistence of infectious pancreatic necrosis virus in Atlantic salmon. *Vet Immunol Immunopathol* 12:359–364
- McVicar AH (1987) Pancreas disease of farmed Atlantic salmon, *Salmo salar*, in Scotland: epidemiology and early pathology. *Aquaculture* 67:71–78
- McVicar AH (1990) Infection as a primary cause of pancreas disease in farmed Atlantic salmon. *Bull Eur Ass Fish Pathol* 10:84–87
- Munro ALS, Ellis AE, McVicar AH, McLay HA (1984) An exocrine pancreas disease of farmed Atlantic salmon in Scotland. *Helgoländer Meeresunters* 37:571–586
- Murphy TM, Rodger HD, Drinan EM, Gannon F, Kruse P, Kortung W (1992) The sequential pathology of pancreas disease in Atlantic salmon farms in Ireland. *J Fish Dis* 15:401–408
- Raynard RS, Houghton G (1993) Development towards an experimental protocol for the transmission of pancreas disease of Atlantic salmon *Salmo salar*. *Dis aquat Org* 15:123–128
- Saint-Jean SR, Minondo MPV, Palacios MA, Prieto SP (1991) Detection of infectious pancreatic necrosis in a carrier population of rainbow trout, *Oncorhynchus mykiss* (Richardson), by flow cytometry. *J Fish Dis* 14:545–553
- Swanson RN, Gillespie JH (1982) Isolation of infectious pancreatic necrosis virus from the blood and blood components of experimentally infected trout. *Can J Fish Aquat Sci* 39:225–228
- Yamamoto T, Clermont TJ (1990) Multiplication of infectious haematopoietic virus in rainbow trout following immersion infection: organ assay and electron microscopy. *J aquat Anim Health* 2:261–270
- Yu KKY, MacDonald RD, Moore A R (1982) Replication of infectious pancreatic necrosis virus in trout leucocytes and detection of the carrier state. *J Fish Dis* 5:401–410